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Mechanism of hypoglycaemic action of methylenecyclopropylglycine

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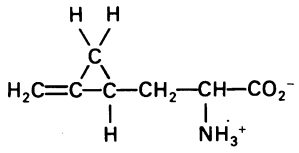
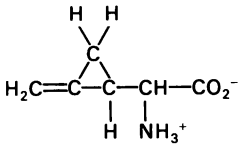
The effects of methylenecyclopropylglycine (MCPG), the lower homologue of hypoglycin A, on starved rats are described. Upon oral ingestion of MCPG (43 mg/kg), a 50% decrease in blood glucose compared with controls was observed after 4 h. The plasma concentrations of lactate and non-esterified fatty acids were substantially increased during this period. The activity of general acyl-CoA dehydrogenase from isolated rat liver mitochondria was not significantly changed. By contrast, the activity of 2-methyl-(branched-chain)-acyl-CoA dehydrogenase decreased by over 80%. The enzyme activity of enoyl-CoA hydratase (crotonase) from pig kidneys decreased by 80% on incubation with the hypothetically toxic metabolite of MCPG, methylenecyclopropylformyl-CoA. These results suggest that the inhibition spectrum of MCPG is quite different from that of hypoglycin A and that similar physiological effects might result from inhibition of different enzymes of β-oxidation, e.g. hypoglycaemia and lacticacidemia. Accumulation of medium-chain acyl-CoA thioesters is probably at the origin of disturbances in pyruvate metabolism.

INTRODUCTION

Methylenecyclopropylglycine (MCPG) was first isolated in 1962 by Gray & Fowden [1] from the kernels of litchi fruit. It is the lower homologue of the better known hypoglycin A, with which it shares the methylenecyclopropyl function:



Hypoglycin A



Methylenecyclopropylglycine (MCPG)

Hypoglycin A induces a strong hypoglycaemia in mammals [2–5]. The mechanism of this effect has been elucidated in some detail, the salient point being an irreversible inhibition of β-oxidation which, in turn, results from a covalent and irreversible reaction of methylenecyclopropylacetyl-CoA (MCPA-CoA) with the flavin of general acyl-CoA dehydrogenase (GAD, EC 1.3.99.3) [6–13]. MCPA-CoA is formed by degradation of hypoglycin A through α-oxidation and subsequent oxidative decarboxylation and conjugation to CoA by the branched-chain oxo-acid dehydrogenase [14]. MCPA-CoA derived from hypoglycin A is therefore the toxic metabolite (mainly) responsible for the observed effects.

Gray & Fowden reported that MCPG has a comparable hypoglycaemic effect on mice [1]. This observation has never been followed up. Recently, we succeeded with the chemical synthesis of MCPG [15] and were also able to obtain sufficient quantities of natural MCPG to study the molecular mechanism of hypoglycaemia induced by MCPG.

MATERIALS AND METHODS

Acyl-CoA thioesters and enoyl-CoA hydratase (crotonase, EC 4.2.1.17) from bovine liver were from Sigma. Crotonyl-CoA was obtained from P-L Biochemicals. ^3H_2O (sp. radioactivity 5 Ci/ml) was obtained from Amersham; hexokinase, glucose-6-phosphate dehydrogenase and L-lactate dehydrogenase were from Boehringer Mannheim. Crotonase from pig kidney was purified as described elsewhere [16]. Butyryl-CoA dehydrogenase (EC 1.3.99.2) from *Megasphaera elsdenii* was isolated by the method of Engel & Massey [17], and GAD from pig kidneys by the method of Lau *et al.* [18]. MCPA-CoA was synthesized as described by Wenz and co-workers [12], and methylenecyclopropylformyl-CoA (MCPF-CoA) by the method of Boschert [15].

MCPG was isolated and purified as described elsewhere [15] from dried kernels of litchi fruits (*Litchi chinensis*). The latter were provided by Dr. A. Münzhuber (Taipei, Taiwan) and were obtained from litchi fruit canneries. The MCPG used for oral administration to rats was 80% pure and free of hypoglycin A as determined by amino acid analysis. Alanine, glycine and valine were the only amino acids present as contaminants.

Abbreviations used: NEFA, non-esterified fatty acids; GAD, general acyl-CoA dehydrogenase; MCPA-CoA, methylenecyclopropylacetyl-CoA; MCPF-CoA, methylenecyclopropylformyl-CoA; MCPG, methylenecyclopropylglycine.

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It was assumed that they would not affect the results of the experiments in the concentrations used.

Animals

Male rats of the Sprague–Dawley strain (strain 50; Ivanovas, Kisslegg, Germany), weighing 160–200 g were starved for 24 h and then given MCPG orally (0–100 mg/kg). For determination of plasma parameters, blood was taken from the retro-orbital venous plexus after 0, 2 and 4 h. The animals were killed by decapitation, the livers removed and the mitochondria obtained by published procedures [19]; 12 animals each were used for the doses 0–40 mg/kg, and six animals each for the doses 50 and 100 mg/kg.

Standard methods

Plasma glucose concentrations were determined by the hexokinase/glucose-6-phosphate dehydrogenase method [20], and lactate concentration was measured with lactate dehydrogenase [20]. Non-esterified fatty acids (NEFA) were extracted from the serum as Cu^{2+} salts by using chloroform, the concentration being determined spectrophotometrically as the diethyldithiocarbamate complex [21]. For statistical comparisons, Student's *t* test was used.

Preparation of [2,3-^{3}H]acyl-CoA thioester

Tritiated iso- and n-butyryl-CoA were prepared from the unlabelled analogues by using bacterial butyryl-CoA dehydrogenase from *Megasphera elsdenii*. Tritiated octanoyl-CoA was prepared by using GAD from pig kidney by the method described elsewhere [22], with the following modifications. The reaction was stopped by addition of sodium deoxycholate (final concn. 0.05%, w/v) at 0 °C, the reaction mixture was applied to a Dowex 1X8 column (20–50 mesh; bed volume 1 ml) and the CoA esters were eluted with approx. 1.5 ml of 1 M-NaCl. Specific radioactivities were: 68500 d.p.m./nmol for octanoyl-CoA, 47900 d.p.m./nmol for butyryl-CoA, and 14600 d.p.m./nmol for isobutyryl-CoA.

The ^{3}H-release assay was carried out analogously to that of Rhead and co-workers [23].

Assay for crotonase

For the study of inhibition of crotonase by MCPF-CoA, 50 μl of crotonase (0.28 μM) was incubated with different amounts of MCPF-CoA in a final volume of 270 μl. The residual activity was measured in samples containing 10 mM-phosphate, pH 7.0, 0.4 M-KCl and crotonyl-CoA (final concn. 73 μM). The decrease in A_{280} was monitored by an Uvicon 820 spectrophotometer. Initial rates of hydration were determined at 280 nm by using a molar absorption coefficient of 3600 litre·mol^{-1}·cm^{-1} [24]. The concentrations of MCPA-CoA and MCPF-CoA were calculated by using a molar absorption coefficient of 15400 litre·mol^{-1}·cm^{-1} at 260 nm. Concentration of crotonase was based on subunits. The enzyme was kept in standard buffer [0.1 M-potassium phosphate, pH 6.3, 10% (v/v) glycerol, 5 mM-mercaptoethanol, 0.3 mM-EDTA].

RESULTS

Modification of blood parameters

The dose- and time-dependent changes in glucose, lactate and NEFA induced by oral administration of MCPG are shown in Table 1. No significant effect was observed at a dose of 25 mg/kg, whereas on administration of 50 mg/kg the blood glucose decreased to approx. 20% of its initial value within 2–4 h as compared with control rats. The calculated ED_{50} value, i.e. that giving 50% decrease in blood glucose, is approx. 43 mg/kg. On the other hand, the lactate concentration increased from 0.88±0.10 to 4.84±0.40 mM during the same period of time. In addition, MCPG gives a large increase in NEFA concentration, from 0.50±0.08 to 2.20±0.25 mM.

Activities of acyl-CoA dehydrogenases

The results summarized in Fig. 1 show that the activity of the major enzyme of β-oxidation, medium-chain acyl-CoA dehydrogenase (GAD), remains essentially unchanged. This is consistent with studies carried out with homogeneous GAD from pig kidney [15]. The latter

Table 1. Dose- and time-dependent changes in blood glucose, lactate and NEFA concentrations after oral administration of MCPG to 24 h-starved rats

Values are means±S.E.M.; *P* values refer to the 4 h value (n.s., not significant).

Dose (mg/kg)	Parameter	Concn. (mM) 0 h	2 h	4 h	Significance
0	Glucose	4.03±0.19	4.24±0.23	4.34±0.17	
	Lactate	1.25±0.30	0.92±0.20	0.88±0.10	
	NEFA	0.69±0.21	0.42±0.18	0.50±0.08	
25	Glucose	4.26±0.21	4.18±0.10	3.85±0.26	n.s.
40	Glucose	4.20±0.24	4.23±0.25	2.80±0.20	$P < 0.01$
	Lactate	0.84±0.20	0.79±0.23	1.57±0.10	$P < 0.001$
50	Glucose	3.96±0.30	4.34±0.22	1.21±0.34	$P < 0.001$
	Lactate	1.13±0.40	1.05±0.20	4.84±0.40	$P < 0.001$
	NEFA	0.31±0.12	1.65±0.24	2.20±0.25	$P < 0.001$
100	Glucose	3.90±0.21	3.90±0.41	1.27±0.41	$P < 0.001$
	Lactate	1.50±0.26	1.20±0.30	4.30±0.32	$P < 0.001$
	NEFA	0.36±0.14	1.62±0.08	2.32±0.11	$P < 0.001$

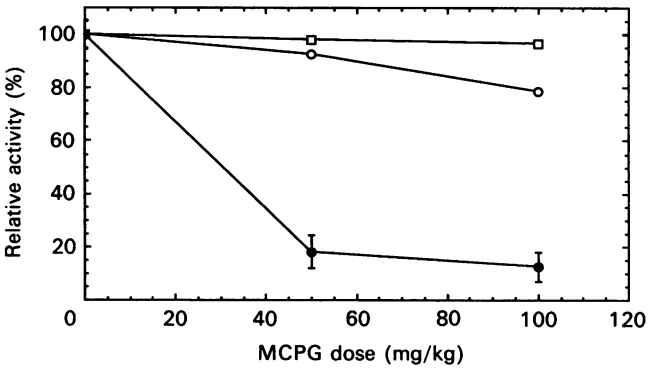

Fig. 1. Activities of acyl-CoA dehydrogenases upon administration of MCPG

The activities of GAD (□), butyryl-CoA dehydrogenase (○) and 2-(methyl)-branched-chain-acyl-CoA dehydrogenase (●) were measured in rat liver mitochondria isolated 4 h after administration of the doses of MCPG. The values given are means (±S.E.M.) from two determinations per liver obtained from two animals. The range of S.E.M. values is shown representatively for 2-(methyl)-branched-chain-acyl-CoA dehydrogenase.

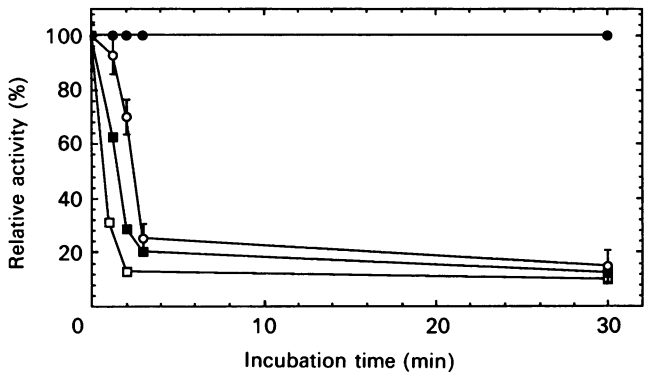

Fig. 2. Dependence of activity of pig kidney crotonase on time of incubation with different amounts of MCPF-CoA

Treatments: (●) 200-fold excess of MCPA-CoA; (○) 44-, (■) 220-, (□) 440-fold molar excess of MCPF-CoA as compared with crotonase. Note that MCPA-CoA does not inhibit the enzyme, and that this incubation serves as control. Samples were taken after the incubation times shown and assayed as described in the Materials and methods section. Results are means±S.E.M. for three experiments.

enzyme binds MCPF-CoA, as demonstrated by rapid changes induced in the oxidized flavin spectrum upon incubation with a 100-fold molar excess of the CoA derivative (results not shown) which are typical of the binding of non-reacting CoA derivatives. However, and in sharp contrast with what was observed with MCPA-CoA by Wenz *et al.* [12], no chemical reaction ensues over a period of 2–3 h. This was deduced from the lack of alteration in the flavin chromophore, which normally indicates reduction, and from the activity profile, which did not change significantly. This also contrasts with 2-(methyl)-branched-chain-acyl-CoA dehydrogenase, the activity of which is decreased to 20% of its initial value.

Activities of crotonase

Measurement of crotonase activity in crude rat liver extracts proved unreliable. Therefore, the effects of the presumed toxic metabolites MCPA-CoA and MCPF-CoA were tested with isolated enzymes available to us. When crotonase (0.3 μM) in standard buffer was incubated with a 200-fold molar excess of MCPA-CoA, no loss of activity was observed over a period of 30 min.

When the same type of experiment was carried out with MCPF-CoA, a time- and concentration-dependent inhibition of crotonase activity was observed, as shown in Fig. 2. Remarkably, inhibition was not complete, but appeared to level off, to approx. 20% of its original value. Repetitive exchange of buffer by ultrafiltration did not lead to substantial recovery of crotonase activity. When the test was carried out with commercial bovine liver crotonase, no comparable effect was observed.

DISCUSSION

The effects of MCPG on the rat are indeed comparable with those observed upon administration of hypoglycin A, in that they induce a dose- and time-dependent decrease in plasma glucose (Table 1) [5]. This essentially confirms observations made by Gray & Fowden [1] with mice. In addition, a marked increase in plasma concentrations of lactate and NEFA is observed. The ED_{50} value for the decrease in the blood glucose concentration is 43 mg/kg.

These observations coincide with the assumption that, by analogy with hypoglycin A, MCPG is degraded to a toxic metabolite which directly inhibits β-oxidation, and indirectly gluconeogenesis, possibly by inhibition of pyruvate carboxylase (a key enzyme of gluconeogenesis), by medium-chain acyl-CoA thioesters which accumulate in the mitochondrial matrix when β-oxidation is inhibited [14]. According to Randle *et al.* [25], the inhibition of β-oxidation contributes to the decrease in blood glucose by activating peripheral glucose oxidation. The decrease in plasma glucose can hardly be explained by an inhibition of GAD or (straight-chain) butyryl-CoA dehydrogenase. They are affected only marginally, if at all.

Inhibition of 2-(methyl)-branched-chain-acyl-CoA dehydrogenase, which is normally implicated in the degradation of valine or isoleucine at the site of the respective CoA thioesters, clearly suggests that a thioester of a MCPG metabolite is involved. The likely candidate is MCPF-CoA. Inhibition of crotonase involved in β-oxidation clearly leads to effects qualitatively similar to those observed upon inhibition of GAD or butyryl-CoA dehydrogenase. The mechanism of inhibition of pig kidney crotonase is not yet clear. Both covalent modification of the enzyme active site upon 'suicide inhibition', as for GAD with MCPA-CoA [12,13], or formation of a very tight-binding product from MCPF-CoA, might explain the findings.

The increase in plasma NEFA may result from inhibition of NEFA oxidation and hormonally induced lipolysis owing to the hypoglycaemic effect of MCPG.

The effect on lactate concentrations induced by MCPG contrasts with what has been observed with hypoglycaemic inhibitors of the transport of long-chain fatty acids into the mitochondria (inhibition of carnitine palmitoyltransferase I, EC 2.3.1.21), such as the phenyl-

alkyloxiranecarboxylic acids, which do not lead to an increase in plasma lactate [26,27]. This appears to suggest that the toxic intermediate of MCPG leads to inhibition of oxidative decarboxylation (e.g. pyruvate dehydrogenase) and/or the citrate cycle.

The most striking phenomenon is the difference in inhibition properties on general acyl-CoA dehydrogenase induced by hypoglycin A or MCPG.

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